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Isolation and Cultivation of Blastocyst-Derived Stem Cell Lines From American Mink (*Mustela vison*)

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ABSTRACT Ten embryonic stem (ES) cell lines from mink blastocysts were isolated and characterized. All the lines had a normal diploid karyotype; of the ten lines studied, five had the XX and five had the XY constitution. Testing of the pluripotency of the ES-like cells demonstrated that 1) among four lines of genotype XX, an X was late-replicating in three; both Xs were active in about one-third of cells of line MES8, and analysis of glucose-6-phosphate dehydrogenase revealed no dosage compensation for the X-linked gene; 2) when cultured in suspension, the majority of lines were capable of forming "simple" embryoid bodies (EB), and two only showed the capacity for forming "cystic" multilayer EBs. However, formation of ectoderm or foci of yolk sac hematopoiesis, a feature of mouse ES cells, was not observed in the "cystic" EB; 3) when cultured as a monolayer without feeder, the ES cells differentiated into either vimentin-positive fibroblast-like cells or cytokeratin-positive epithelial-like cells (less frequently); neural cells appeared in two lines; 4) when injected into athymic mice, only one of the four tested lines gave rise to tumors. These were fibrosarcomas composed of fibrobalst-like cells, with an admixture of smooth muscular elements and stray islets of epithelial tissue; (5) when the ES cells of line MES1 were injected into 102 blastocyst cavities and subsequently transplanted into foster mathers, we obtained 30 offspring. Analysis of the biochemical markers and coat color did not demonstrate the presence of chimaeras among offspring. Thus the cell lines derived from mink blastocysts are true ES cells. However, their pluripotential capacities are restricted.

Key Words: Embryonic stem cells, Cell differentiation, Pluripotency

INTRODUCTION

Blastocyst-derived cells can be maintained in vitro for a long time without loss of pluripotency (Evans and Kaufman, 1981; Martin, 1981). The ES cells can, however, differentiate into a wide variety of types when cultured without a feeder layer or injected subcutaneously into syngenic mice (Martin, 1981; Robertson, 1987). Furthermore, when injected into the blastocoele cavity, the ES cells can contribute to the full range of adult tissues, including germ cells, i.e., generate chimaeras (Bradley et al., 1984). Mouse chimaeras thus produced are able to yield progeny carrying genes of ES cell origin (Gossier et al., 1986; Robertson et al., 1986; Hooper et al., 1987).

At present, data on the isolation of the ES cells in species other than the mouse are available. Handyside et al. (1987) have described primary cultures from early embryos of sheep; these, however, died after continued passage. ES-like permanent porcine lines were established by transformation with polyoma virus (McWhir, 1988). Piedrahita et al. (1990) have isolated porcine and ovine ES-like cells with restricted pluripotential capacities, and they were unable to generate porcine chimaeras. Also, Evans et al. (1990) and Notarianni et al. (1990) have isolated permanent bovine and porcine ES-like cell lines from early embryos. Detailed testing of the pluripotency of these cell lines, as well as the production of chimaeras, are certainly needed.

This paper is concerned with isolation and characterization of blastocyst-derived cell lines from mink. The results of pluripotency tests in vivo and in vitro allowed us to conclude that the mink blastocyst-derived cell lines are true ES cells, though with restricted pluripotential capacities.

MATERIALS AND METHODS

Animals

Standard (wild type, genotype +/+) and silver-blue (homozygous for the recessive mutation platinum, genotype p/p) minks were bred at the experimental farm of this Institute. Minks of both genotypes were used as donors of blastocysts.

Embryos and Establishment of ES-Like Cell Lines

Ninety-two and 25 embryos were removed in 1990 and 1991, respectively, from naturally mated minks 8–10 days postcoitum. Blastocysts were collected by

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TABLE 1. Treatment of Mink Blastocysts and Their In Vitro Culture Conditions

Blastocysts		Culture conditions					
		With feeder				With no feeder but in the presence of LIF	
						Plastic plate	
Genotype	Treatment	FMV[a]	MV[a]	MEF[a]	MF[a]	Gelatinized	Non-gelatinized
+/+	Zona pellucidae removal N = 49	4	6	13	—	10	16
	N = 3[b]	—	—	—	3	—	—
	Immunosurgery N = 18	—	—	—	—	18	—
p/p	Zona pellucidae removal N = 20	5	—	15	—	—	—
	N = 22[b]	—	—	—	22	—	—
	Immunosurgery N = 5	—	—	5	—	—	—

[a]FMV, MV, MEF, and MF are feeder layers: FMV, an established mink fibroblast-like cell line derived from a newborn mink in 1987; MV, an established mink fibroblast-like cell line derived from a newborn mink in 1979; MEF, primary mouse embryonic fibroblasts; MF, primary mink embryonic fibroblasts.
[b]Experiments performed in 1991.

flushing the oviduct with buffer PB1 (Hogan et al., 1986). Early and middle blastocysts were selected for further manipulation. Zona pellucidae of 94 blastocysts were removed by treatment in 0.5% pronase solution (Table 1).

Forty-three blastocysts in 1990 and 25 in 1991 were cultured in "embryo medium" (α modification of Eagle's MEM, supplemented with 20% fetal calf serum and 0.1 mM β-mercaptoethanol) on γ-irradiated feeder layers at 37°C in a humidified 5% CO_2 atmosphere. Medium was changed every 2 days.

The following cell lines were used as feeder layers: 1) primary mouse embryonic fibroblasts (MEF); 2) primary mink embryonic fibroblasts (derived from mink embryos 30–35 days postcoitum) (MF); and 3) two established mink fibroblast-like cell lines, MV and FMV, derived from newborn minks. Feeder cells were irradiated with 4,000 R (185 R/min).

Twenty-six blastocysts were cultured with no feeder but in the presence of leukemia inhibitory factor (LIF) in "embryo medium" (Table 1). LIF was added to the medium at a concentration of 10^3 units/ml (Williams et al., 1988). Murine LIF was a kind gift from Dr. N.A. Nicola (The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). Blastocysts were cultured in plastic plates. Ten were gelatinized, and 16 were not (Table 1).

Twenty-three blastocysts were subjected to immunosurgery according to Solter and Knowles (1975) for isolation of inner cell masses (ICM) (Table 1). Antimink spenocyte serum was produced in a rabbit that had been bled 10 days following three intravenous injections of 5×10^8 mink splenocytes. The ICMs obtained in this manner were cultured either on feeder (MEF) or with no feeder and in the presence of LIF (Table 1).

Differentiation of Blastocyst-Derived Cells In Vitro

Induction of spontaneous differentiation of the ES-like cells in vitro was carried out essentially as described by Robertson (1987). Mink ES-like cells were stimulated to form "embryoid bodies" (EB) by transfer from the feeder into suspension culture. Several EBs were replaced (after 4 days) on gelatinized plastic plates for further culturing without feeder.

Tumorigenicity in *nude* Mice

Athymic (*nude*) mice were routinely given injections of $1–2 \times 10^7$ ES-like cells suspended in 0.3 ml Eagle's medium at one subcutaneous site per animal.

In Vivo Differentiation of ES-Like Cells

The ability of mink ES-like cells to differentiate in vivo was tested by injection into blastocoels. Recipient blastocysts of genotype p/p were collected 8–10 days postcoitum, and ~15–20 ES-like cells (genotype +/+) were injected into a blastocoele.

Zona pellucidae of mink blastocysts are very resistant and plicable, enfolding under mechanical pressure. We rarely managed to prick them with a microneedle or slender micropipette without damaging the embryo. For this reason, we locally digested the membrane by applying pronase. Approximately 15–20 donor ES-like cells were sucked into a micropipette (diameter 15–20 μm), a tiny bubble of paraffin oil was sucked in, then 15–20 nl 1% pronase solution was drawn into the tip of the micropipette. The micropipette was pressed to the surface of a blastocyst so that it bent slightly. Evidence of local perforation was the reassumed spherical shape of the blastocyst. The volume of pronase and expulsion rate from the micropipette were adjusted so that enzyme digestion to completion would be coincident with the expulsion of the total volume of pronase solution. The micropipette was advanced through the perforation to the ICM site; thereafter, the droplet of paraffin oil and the ES cells were squeezed out.

Injected blastocysts were transferred to recipients. The color phenotype of the offspring was recorded at birth. The phenotypic expression of peptidases B and D

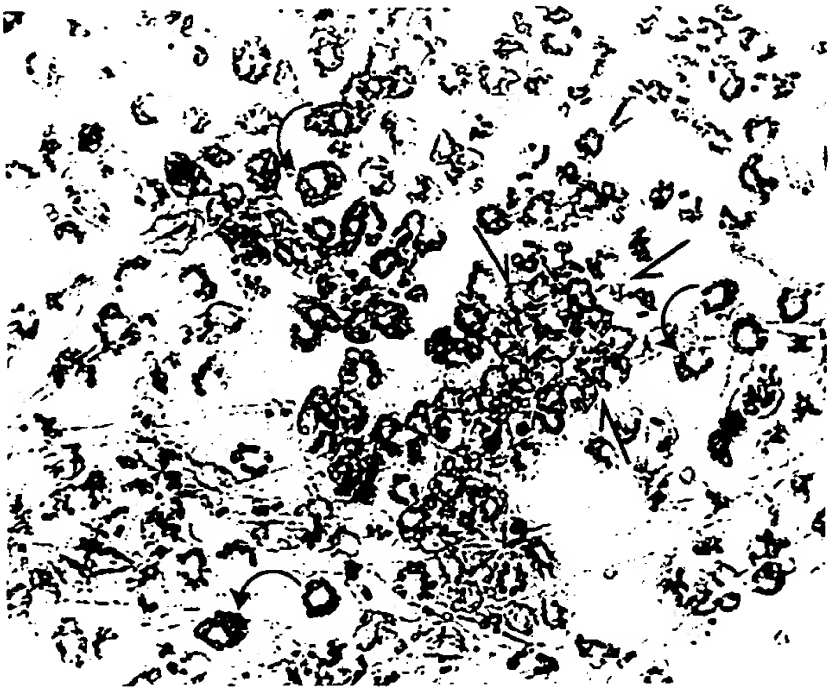

Fig. 1. Blastocyst spread 3 days after its attachment to feeder, MF cells. A group of small cells near the center is presumably a derivative of the ICM (straight arrows); giant cells of trophoblast origin are visible at the periphery (curved arrows). ×125.

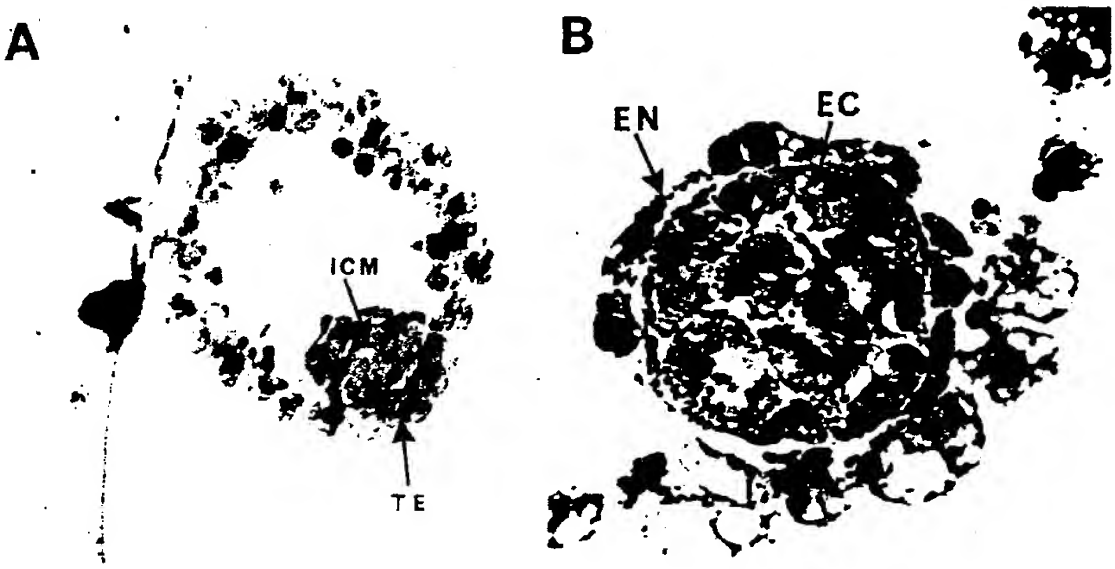

Fig. 2. Mink embryo (genotype +/+) at the early blastocyst stage. The ICM is composed of epithelial cells of the primary ectoderm (EC) with nuclei containing one or two nucleoli and cytoplasm with conspicuous granular content. A layer of visceral endoderm (EN) is seen in the ICM. TE, trophoectoderm. A, ×125. B, ×1,250.

was examined twice, at the ages of 2 and 3 months. Both enzymes were assayed in red blood cells and the tail tip in all offspring. The expression of peptidases B and D in liver, kidneys, skeletal and heart muscles, spleen, and brain was also studied in three minks that died 2 days after birth.

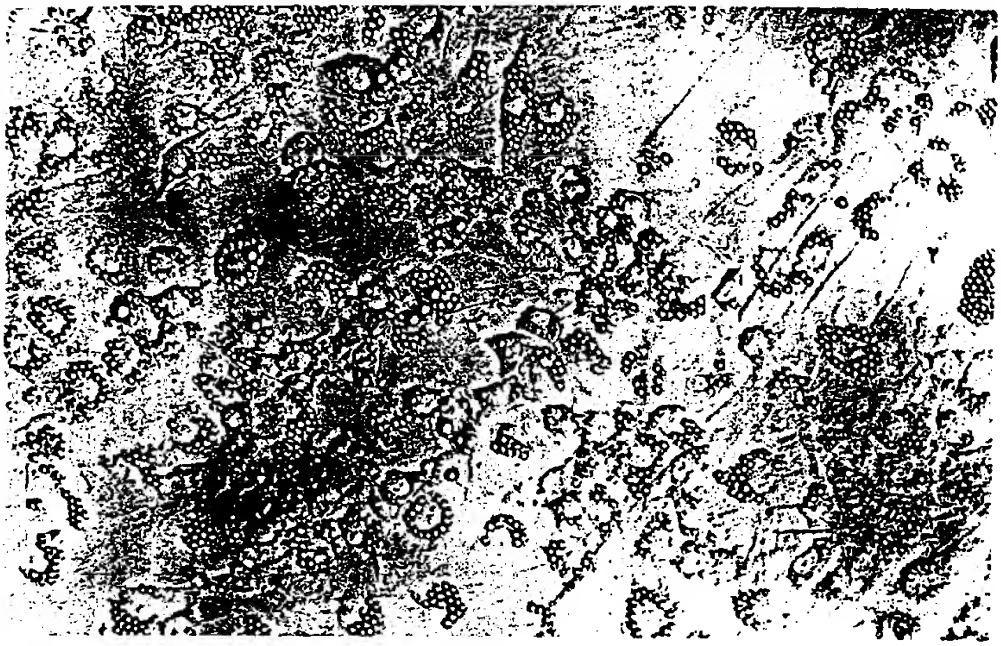

Fig. 3. Morphological appearance of the ES-like cells, MES1 cells, viewed under the interference microscope on the next day after seeding. ×500.

Light Microscopy

The EBs from suspension cultures were fixed for 2 hr in cold 2.5% glutaraldehyde in phosphate buffer, post-fixed in 1% osmium tetroxide buffered with phosphate, dehydrated in increasing concentrations of ethanol, and embedded in Epon. Semithin sections (1–2 μm) were obtained with an Reichert OmU2 ultramicrotome and stained with fuchsin.

Mink blastocysts were flushed from oviducts with saline phosphate buffer. Zona pellucidae were removed with 0.5% pronase solution. Fixation and embedding in Epon were essentially as described above for the EB. Specimens of tumors dissected from athymic mice were fixed in Bouin's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Immunofluorescence Microscopy

Monolayer cultures of ES cells growing on plastic or on coverslips were washed in saline phosphate buffer and prefixed in 0.4% formalin for 10 min. Prefixative contained 0.1% Triton X-100 in saline phosphate buffer. Monolayers were further treated with 0.5% Triton X-100 for 15 min, refixed in 2% formalin for 10 min, treated with 1% ovalbumin for 20 min, and incubated with primary and secondary antibodies in a humid chamber for 45 min. Monoclonal antibodies against four classes of intermediate filament proteins were used to detect neurons (antineurofilament; a kind gift from Dr. I. Prudovsky, Institute of Molecular Biology, Moscow), epithelial cells (anticytokeratin; a kind gift from Dr. S. Vasetsky, Institute of Developmental Biology, Moscow), muscle (anti-α-actinin; Institute of Cytology, St. Petersburg), and mesoderm derivatives (antivimentin; Sigma, St. Louis, MO). Also, monoclonal antibodies against enolase-B were used to detect neurons (antienolase-B; a kind of gift from Dr. I. Prudovsky). The primary antibodies were used at a concentration of 10 μg/ml. Secondary antibodies were pig antimouse immunoglobulins conjuated with fluorescein isothiocyanate (Sevac, Czechoslovakia). After staining, the preparations were placed on glass slides in a 1:1 glycerol/saline phosphate buffer solution.

Karyotype Analysis

Metaphase spreads were prepared and G-banded using the trypsin-Giemsa staining method (Sukoyan et al., 1984). Mink karyotypes were analyzed according to the nomenclature of Mandhal and Fredga (1975).

The presence of a late-replicating X chromosome in mink ES-like cells was identified according to Kanda (1973) or with the use of bromodeoxyuridine (BrdU) incorporation as described by Alves and Jonasson (1978) and by McBurney and Stutt (1980). The culture of the ES-like cells was supplemented with BrdU at a concentration of 30 μg/ml 6.5–8 hr before fixation.

Analysis of Biochemical Markers

Electrophoresis in 14% starch gel of the variants of peptidases B and D (PEPB and PEPD) has been described elsewhere (Mullakandov et al., 1986). Activity of the X-linked marker glucose-6-phosphate dehydrogenase (G6PD) in the extracts of the ES-like cells was measured by the method of Dao et al. (1979). Specific

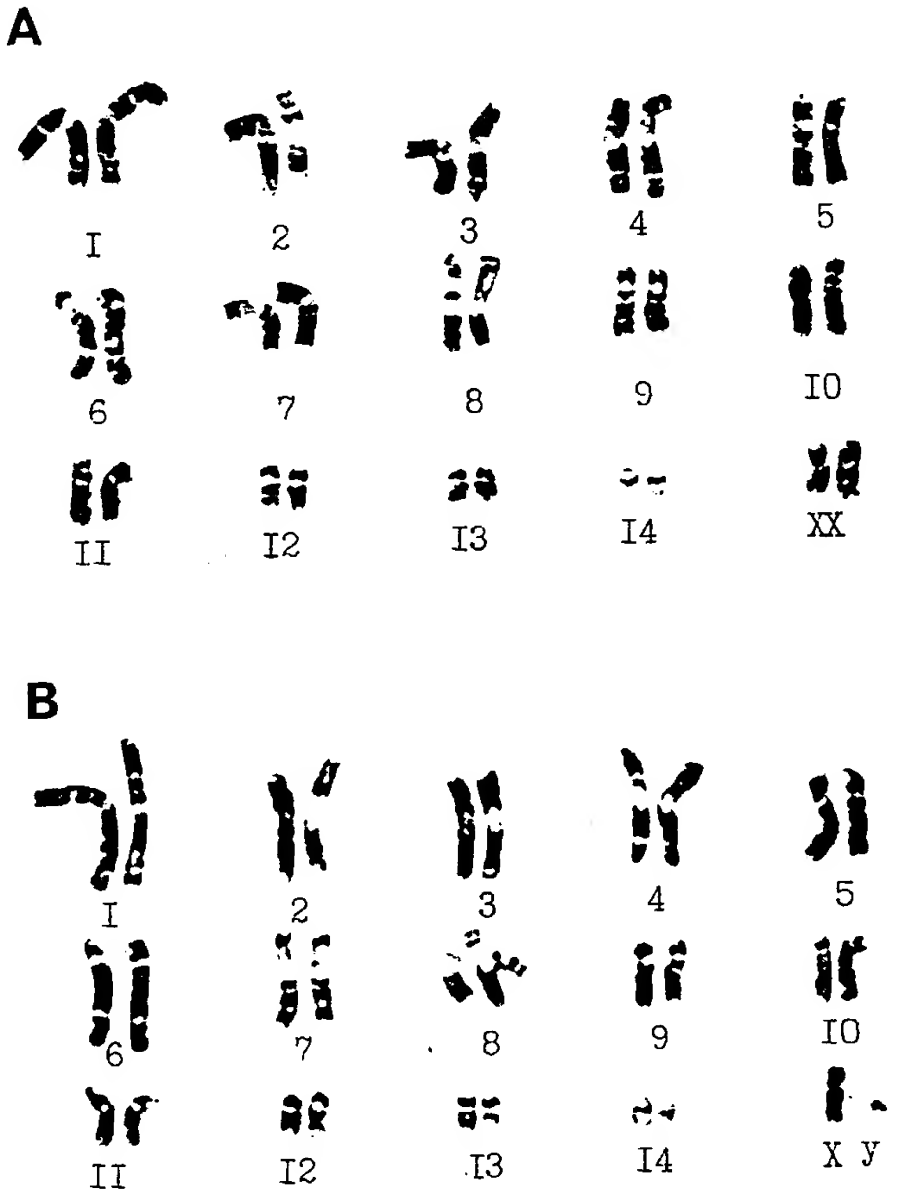

Fig. 4. G-banding patterns of the chromosomes of the ES-like cells, genotype XX (MES4 cells) at passage 8 (A) and genotype XY (MES2 cells) at passage 11 (B).

activities were expressed as nanomoles of product formed per minute per million cells.

RESULTS

Establishment of Blastocyst-Derived Cell Lines

Of 26 explanted blastocysts, genotype +/+, seven attached to the feeder 3–15 days after seeding in vitro. During the next 2–3 days, formation of primary outgrowths was observed near the attached blastocysts. These outgrowths had an epithelial-like morphology, and they grew like monolayer cells. The attached blastocysts, occasionally, spread into a monolayer, and the cell populations were represented by at least two types in such spreads: 1) large cells containing large granular cytoplasm, presumably trophoblast cells (Fig. 1) and 2) small cells with high nucleus to plasm ratios; these tended to group (Fig. 1). The cells superficially resembled the ICM cells, and this resemblance suggested their derivation from the ICM. Figure 2 shows the morphological appearance of a mink embryo at the early blastocyst stage. As can be seen in Figure 2B, the ICM

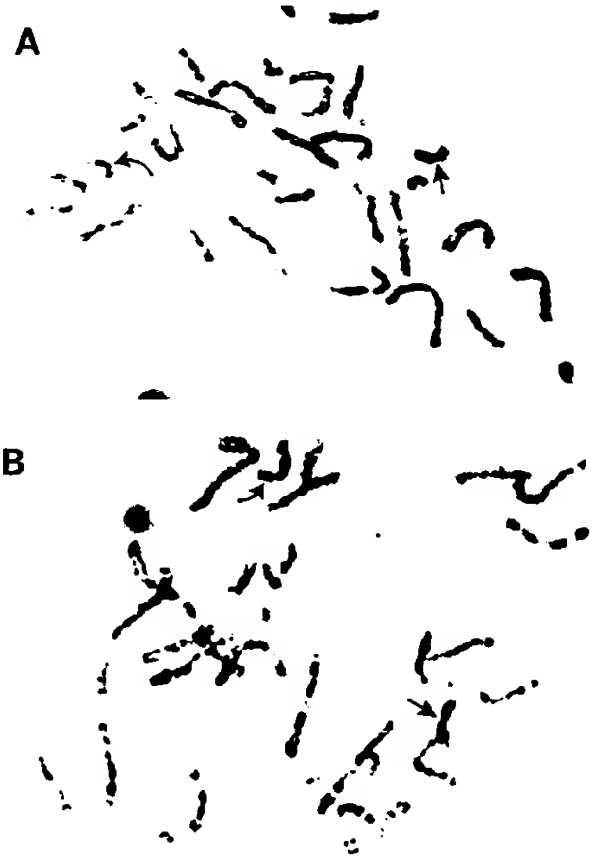

Fig. 5. Late-replicating X chromosome in MES10 cells detected by two methods, those of Kanda (1973) (A) and McBurney and Strutt (1980) (B). A dark-staining late-replicating X (straight arrows) and a light-staining synchronous-replicating X (curved arrows) are shown.

consists of cells of the primary ectoderm and visceral endoderm. The cells of the primary ectoderm are represented by cells showing epithelial-like morphology with a prominent nucleus and one or two nucleoli having granular cytoplasm (Fig. 2B). The ICM and cells in the spread blastocyst are quite similar (Figs. 1, 2B). It should be emphasized, however, that the population of the presumptive ICM cells in the primary outgrowths has no distinct boundary delineating them.

The primary outgrowths disaggregated 7–10 days after their appearance, and they were placed on fresh feeder. Fast growing colonies were observed. The ES-like cells show morphological similarity to the ICM cells. Figure 3 presents the morphology of the cells viewed under the interference microscope.

Primary outgrowths from the blastocysts of genotype +/+ yielded six independent ES-like cell lines (five in 1990 and one in 1991) designated as the *MES* series (one of the ES-like cell colony died from bacterial contamination) (Table 1). MES1, MES2, MES4, and MES10 were derived from different animals, whereas MES3 and MES5 cell lines arose from different blastocysts of a single mink embryo.

The ES-like cell lines were isolated by utilizing different feeders: MV for MES1; FMV for MES4; MEF for MES2, MES3 and MES5; and MF cells for MES10 lines. However, after passages 10–12, all the MES cell lines were cultured on the MF cells. These feeders were found to be best for maintenance of the MES lines in the undifferentiated state.

In 1990, no formation of ES-like cell colonies was observed during culturing of 20 blastocysts of genotype p/p (Table 1); in 1991, however, six ES-like cell colonies formed from 22 blastocysts of this genotype (Table 1). Of these, four yielded MES6, MES7, MES8, and MES9 lines. The dramatic difference in outgrowth abilities of the blastocysts of genotype p/p in different experiments may be explained by the effects of feeders: FMV and MEF served as feeders in the first experiment and MF cells in the second experiment (Table 1).

No ES-like cell colonies, not even attachment to the substratum, were observed during culturing of blastocysts (genotype +/+) in the presence of LIF without feeder (Table 1). Also, no colonies were observed when the ICMs were cultured with or without feeder (Table 1).

G-Banding Chromosomes and the Late-Replicating X Chromosome in the ES-Like Cell Lines

Analysis of the G-banded chromosomes of the MES series identified in ten lines: five of genotype XX (MES4, MES5, MES8, MES9, and MES10) and five that were genotypically XY (MES1, MES2, MES3, MES6, and MES7) (Fig. 4A,B). All the MES lines had a diploid chromosome set, 2N = 30, at passages 8–15. There were no visible chromosomal rearrangements in the MES cells at the same passages (Fig. 4A,B). Also, repeated cytogenetic analysis of the MES1 and MES8 cells at passage 30 showed that they retained their diploid chromosome set, with no rearrangements.

Analysis of three lines (MES4, MES9, and MES10) of the ES-like cells of genotype XX at passages 8–15 demonstrated that 60–70% of the cells contained a dark-staining X chromosome (Fig. 5A,B). The estimate is close to that obtained by analysis of the late-replicating X in the fibroblast-like cells (B7 line) of genotype XX (Table 2). Kanda (1973) has observed the same level of late-replicating X in somatic cell lines of genotype XX. This suggests that the ES-like cell lines may have one of the Xs inactive.

As the data of Table 2 show, the late-replicating X is identified in only 40–45% of cells in line MES8, thereby indicating that approximately one-third of these cells have both Xs in an active state. It is of pertinence to our data that the presence of a late-replicating X in cultures of superficially undifferentiated teratocarcinoma cells was occasionally not associated with dosage compensation of the genes located in the X chromosome (McBurney and Adamson, 1976; Paterno et al., 1985). With this in mind, we analyzed the activity of the X-linked marker G6PD in lines MES6 (genotype XY) and MES8 and MES10 (genotype XX). The results dem-

TABLE 2. Proportion of Metaphases in Mink ES-Like Cells (Genotype XX) Containing the Dark-Staining X Chromosome

Cell line	Passage number	Method	Number of metaphases examined	Number of metaphases with the dark-staining X chromosome	Percentage of cells with the late-replicating X chromosome
MES4	16	BrdU-incorporation	41	25	61
MES8	9	Kanda	71	29	41
		BrdU-incorporation	54	24	47
MES9	10	BrdU-incorporation	54	32	59
MeS10	10	Kanda	43	25	58
	11	BrdU-incorporation	56	30	53
B7[a]	250	Kanda	59	33	56
		BrdU-incorporation	47	29	62

[a]An established mink fibroblast-like cell line with diploid chromosome set (genotype XX).

onstrated that the activity of G6PD is the same in MES6 and MES10 (15.6 ± 4.3 and 21.2 ± 1.9 nM per minute per million cells, respectively), whereas its activity in MES8 (41.6 ± 2.9 nM per minute per million cells) is twofold that in MES6. These results reassured us that the presence of a late-replicating X in MES10 is associated with dosage compensation of the G6PD gene, whereas its presence in some MES8 cells is not associated with the effect.

Differentiation of Mink ES-Like Cells In Vitro

Two or three days after transfer of the ES-like cells to the suspension culture, there appeared a vesicular formation of an EB (Fig. 6A,B). At the first steps, the EB had a monolayer wall (Fig. 6A); after continued culturing, there appeared an EB with a multilayer wall (Fig. 6B) but in several MES lines only (for instance, MES1 and MES8). The morphological appearance of an EB of type 1 is shown in Figure 7A,B. It can be clearly seen that they are composed of endoderm-like cells, which form a membrane-like structure. These "simple" EBs frequently give outgrowths of new EBs similar in morphological appearance.

An EB of the second type, "cystic," may be encountered along with endoderm-like cells resembling mesenchymal (Fig. 8A,B). However, formation of ectoderm-like cells or foci of yolk sac hematopoiesis, the feature of mouse EB, was not observed in mink "cystic" EB. It should be noted that "cystic" EB never appeared in tested MES2, MES4, MES7, and MES10, whereas almost one-half of the EBs was of "cystic" during differentiation of MES1 and MES8.

The EBs disaggregated 7–10 days after their appearance, and they were placed on gelatinized plastic plates in embryo medium without feeder. These cells underwent further differenatiation. Figure 9 shows the expression of vimentin in differentiating cells at different times after their transfer to culture with no feeder. It is clearly seen that at the early stages (3 days after seeding) only few cells are positive for vimentin and that 3–4 weeks after culturing the majority of the cell popu-

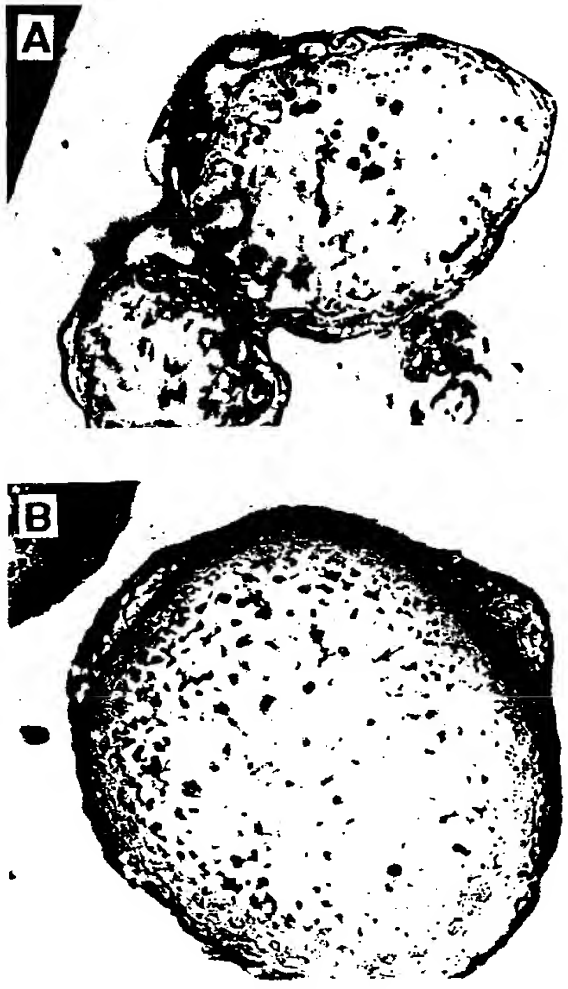

Fig. 6. EBs arisen from MES2 (A) and MES1 (B) after their culturing in suspension. A: "simple" EB arisen 4 days after culturing. Note the monolayer wall and a "daughter" body formed from the large EB. B: a "cystic" EB arisen 14 days after culturing. A multilayer wall is seen. A, ×312. B, ×125.

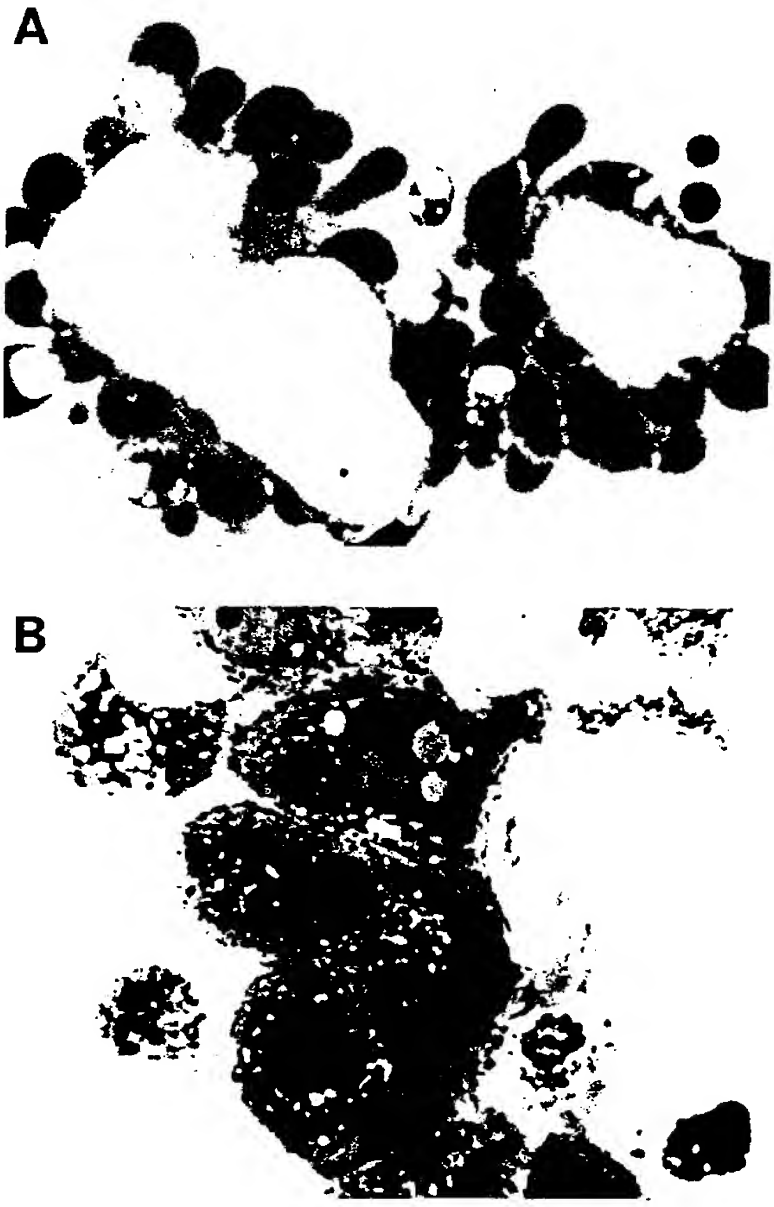

Fig. 7. Morphology of a "simple" EB arisen 4 days after placement of MES1 cells in suspension culture. The EB has a distinct monolayer wall composed of typical endoderm-like cells. A, ×250. B, ×1,250.

lation contains the intermediate filament (Fig. 9A,B). It should be noted that bright fluorescence was observed in fibroblast-like cells, whereas epithelial-like cells fluoresced weakly.

Taken together, the results with the use of antibodies against cytokeratin were the same (Fig. 10A,B). Indeed, it can be seen that on day 1 only few cells were weakly positive for the antigen, whereas the number of positive cells at the later stage of induced differentiation was greater (Fig. 10A,B).

We rarely observed fibrillar structures resembling muscle fibers in the differentiating population of the ES-like cells. However, when antibodies against α-actinin were used, these structures fluoresced very weakly (data not shown).

MES8 and MES9 were unique among the other examined lines of the MES series. In in vitro differentiation induction, we observed frequently neuron-like cells 30–50 days after seeding of ES-like cells in culture without feeder. The neuron-like cells frequently formed an intricate network. They started to appear 25–30 days after culturing of MES8 and MES9 cells with no

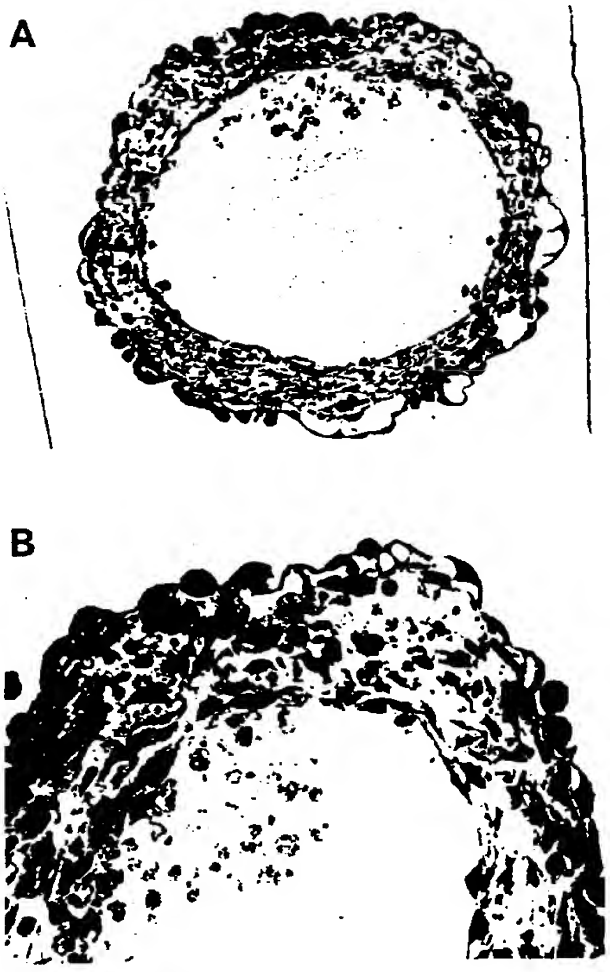

Fig. 8. Morphology of a "cystic" EB arisen 12 days after placement of MES1 cells in suspension culture. The EB is composed of a multilayer wall, with the external layer consisting of endoderm-like cells and the internal layer of mesenchyme-like cells. A, ×125. B, ×500.

feeder. Figure 11 illustrates positive fluorescence in the neuron-like cells when we used antibodies against enolase-B, a specific marker of neural cells. The same results were obtained when we used antibodies against neurofilament. Thus the data suggest that, at least, two ES-like cell lines, MES8 and MES9, are able to differentiate and give rise to neuron-like cells.

The ES-like cell lines examined gave rise, as a rule, to a small number of differentiated derivatives. In most lines examined, neither cells of neuroglial type (except the MES8 and MES9) nor epithelial cells of secretory or keratin type were ever observed. This paucity in the differentiation spectrum may be evidence of restricted pluripotency of mink ES-like cell lines or inappropriateness of in vitro conditions for normal differentiation.

In Vivo Differentiation of Mink ES-Like Cells

After subcutaneous injection of ES-like cells of MES1 line, but not of MES2, MES4, and MES8 lines, into athymic mice, we observed the appearance of rapidly growing tumor on days 12–15. The morphological appearance of some tumors is shown in Figure 12A,B. The tumors are typical fibrosarcomas composed mainly of fibroblast-like cells (Fig. 12A,B). However, rare islets of epithelial tissues consisting of atypical epithelium of granular type were found scattered about as well (Fig. 12B). There were also stray islets of smooth, even striated, muscle. There was not a single tumor with keratin epithelium or neuroglial cells. The variety of cell types in the examined tumors was small. Once again, this supports the suggestion that the pluripotency of the mink ES-link cells may be restricted.

In 1991, we performed the first experiment with the injection of ES-like cells into the blastocoele cavity. The ES-like cells of MES1 line served as donors. The number of transplanted blastocysts of genotype p/p was 102 after injection of the ES-like cells (Table 3). The blastocysts produced 30 pups, and, of these, 22 are presently living. The offspring are nonchimeric, as judged by the results of analysis with the biochemical markers PEPB and PEPD as well as by coat color (Table 3).

DISCUSSION

According to our observations, 3–15 days elapsed from the time of in vitro plating of mink blastocysts to their attachment to the feeder. The blastocysts increased in size during this period. We know nothing about the processes concerned with the commitment and differentiation proceeding in the "floating" blastocysts. What could be expected is that, the earlier a "floating" blastocyst gets attached to the feeder, the less the mechanism(s) maintaining its pluripotency would be affected.

The morphological observations made during the early formation of the outgrowths suggest that cells of all parts of the embryo are capable of growing in vitro during this period. However, trophoblast cells soon disappear, and they are no longer encountered among the primary colonies of ES cells. At the early stages of the formation of colonies of ES cells, cells morphologically similar to ICM cells are identifiable, although it is impossible to decide whether they have arisen from primary ectoderm or endoderm.

We used several independent approaches to estimate the pluripotency of the ten isolated lines of ES cells. First was the presence of a late-replicating X chromosome as evidence for one of the Xs being inactive. It is well known that X-chromosome inactivation occurs at the blastocyst stage; its first overt manifestation is observed in cells of the trophoectoderm and parietal endoderm and later in the primary ectoderm and visceral endoderm (Grant and Chapman, 1988). Of the four lines of mink ES cells (MES4, MES8, MES9, and MES10), a late-replicating X chromosome was identified in three (MES4, MES9, and MES10) in 60% of cells. This is close to the percentage determined for the line of somatic cells of genotype XX (Kanda, 1973; Table 2). There was thus reason for inferring that one of the X chromosome is in an inactive state in the three lines of

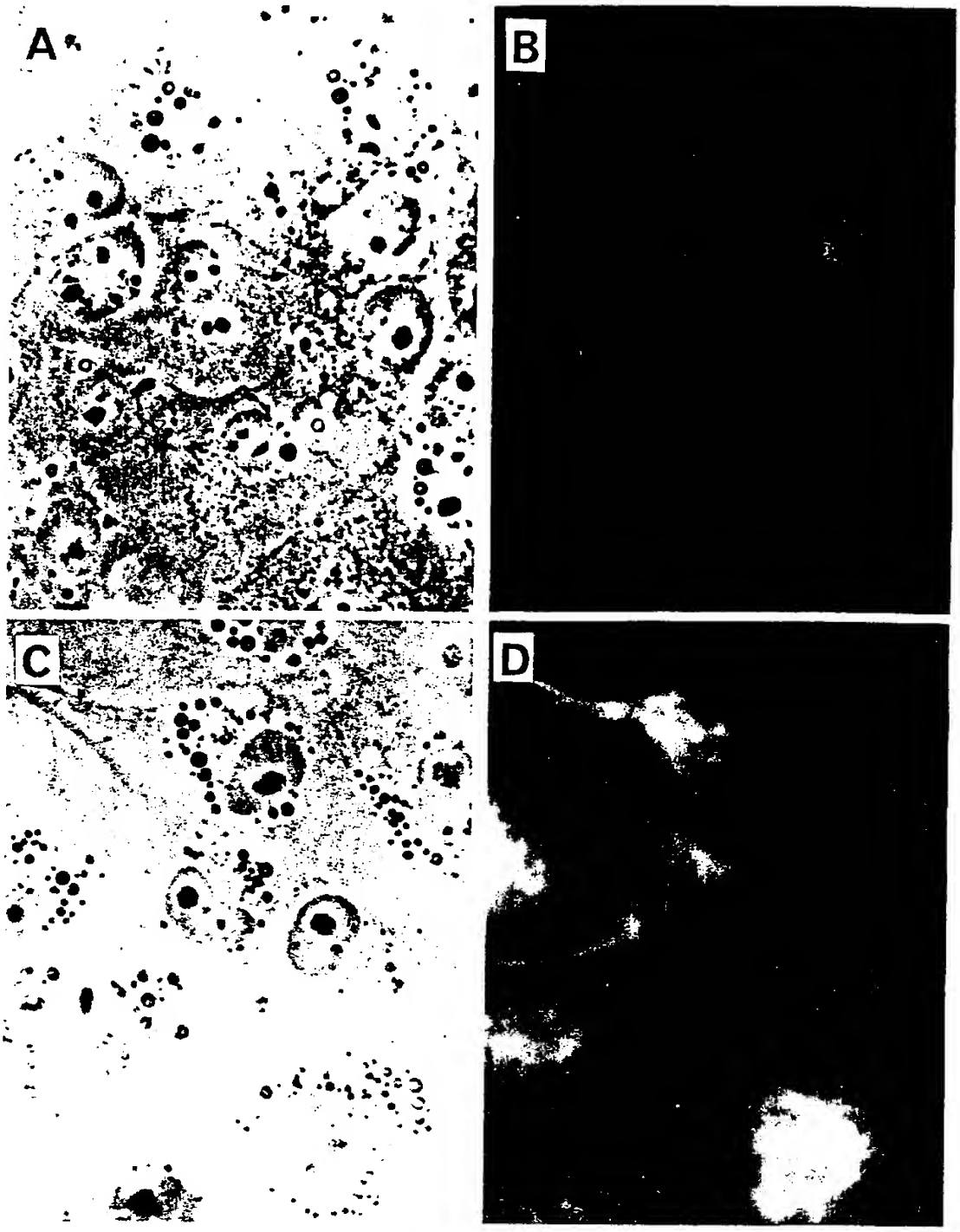

Fig. 9 Expression of vimentin filaments in differentiated MES4 cells in the monolayer culture without feeder. A,B: Cells 2 days after seeding. A, phase-contrast microscopy. B, immunofluorescent staining against vimentin, weak fluorescence C,D: Cells 28 days after seeding; C, phase-contrast microscopy. D, immunofluorescence of antibodies against vimentin reveals fluorescing cells. A,C ×700.

ES cells. Support for this inference came from the data on G6PD activity in MES10, which was found to be the same as in MES6 of genotype XY. Our data, however, are at variance with those of Rastan and Robertson (1985), according to which not more than 20% of cells in undifferentiated mouse ES cell lines contain the late-

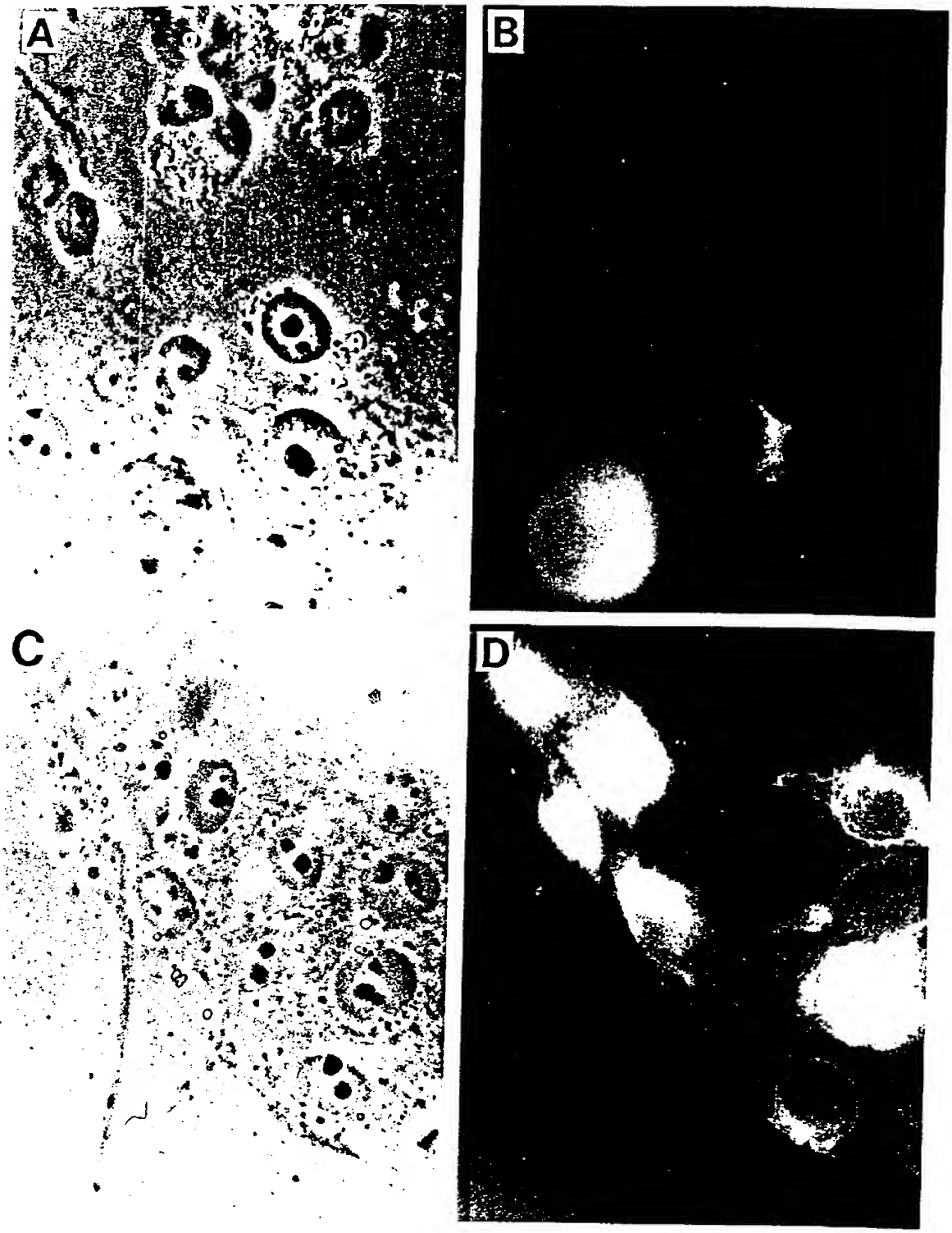

Fig. 10. Detection of cytkeratin in differentiated MES1 cells in the monolayer culture without feeder. A,B: cells 2 days after seeding. A. phase-contrast microscopy. B, immunofluorescent staining against cytokeratin. two cells only fluoresce. C,D: Cells 24 days after seeding. C. phase-contrast microscopy. D. immunofluorescent staining against cytokeratin. A,C ×700.

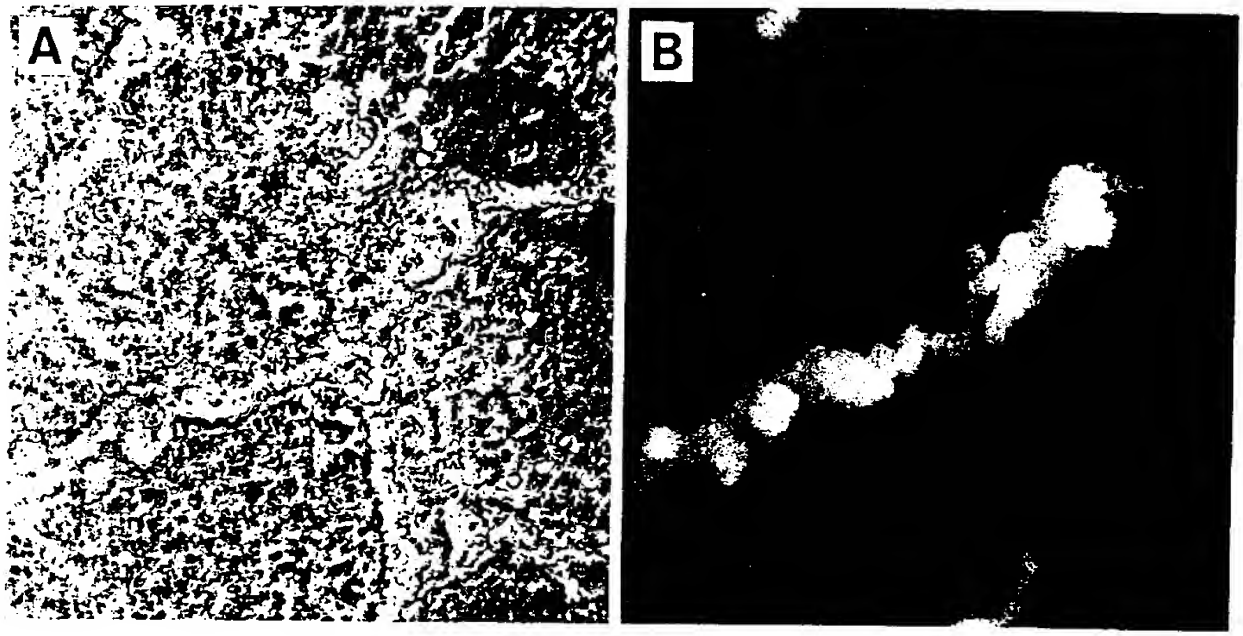

Fig. 11. Neural cells in differentiated MES8 cells 44 days after culturing without feeder. A, phase-contrast microscopy. B, immunofluorescent staining against enolase-B. A, ×300.

replicating X chromosome. Under the effect of induced in vitro differentiation, the percentage of cells with the late-replicating X rose to 40% or more, which meant that the state of the X correlated with the differentiation process (Rastan and Robertson, 1985). It may be concluded that mink ES cells in the three lines (MES4, MES9, and MES10) have passed through the differentiation stage involving inactivation of one of the X chromosomes.

We observed that only 40–45% of cells have a late-replicating X chromosome in line MES8; i.e., in approximately one-third of these cells, both Xs are in an active state. Furthermore, measurements of G6PD activity in MES8 and MES10 cells demonstrated that in the former it is twice as high as in the latter and also in MES6 of genotype XY. Thus although 40% of MES8 cells have a late-replicating X, the fact that there is no dosage compensation of the gene G6PD demonstrates that it is active in all the MES8 cells. Taken together, the data justify the conclusion that MES8 is less committed than the other lines of series MES with genotype XX (MES4, MES9, and MES10).

Second, we observed that the various lines of mink ES cells behave differently when they are placed in suspension culture. All the lines could form "simple" EBs, and, in contrast, only two cell lines, MES1 and MES8, showed the capacity to form multilayer wall EBs ("cystic"). It should be noted that we never observed "true" EBs similar to those described for suspension culture of mouse ES cells (Robertson, 1987). The EB derived from mink ES cells formed neither ectoderm nor structures resembling foci of yolk sac hematopoiesis.

The observations made for in vitro differentiation of mink ES cells grown without feeder also revealed

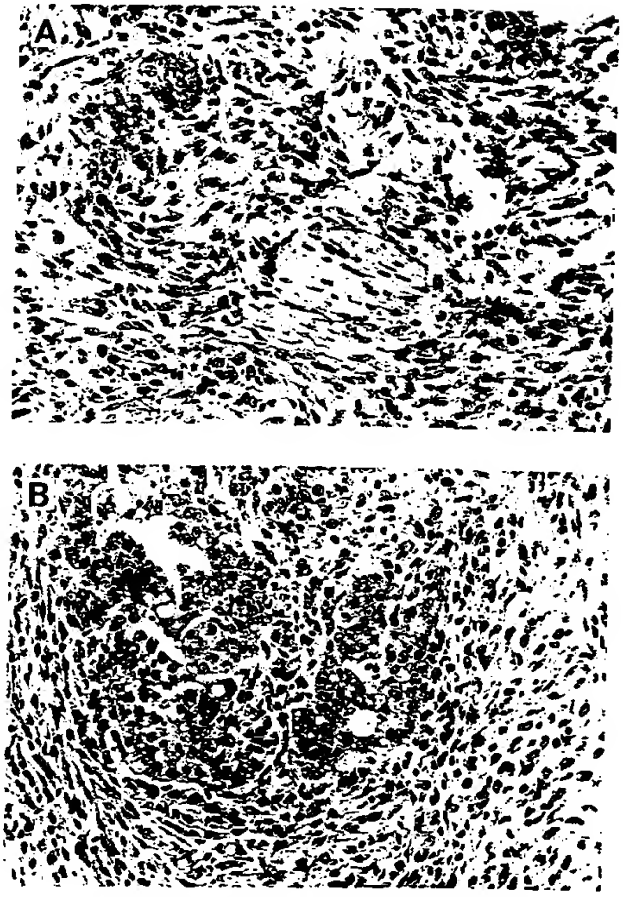

Fig. 12. Morphological appearance of a tumor developed after subcutaneous injection of MES1 cells into athymic mice. A. Illustrative fibrosarcom; near the center, a region of smooth muscle is seen. At left, top, a small islet of epithelial tissue. B. Region of epithelial tissue is prominent in the mass of fibroblast-like cells. ×500.

TABLE 3. Results of Injection of the MES1 Cells Into a Mink Blastocoele of Embryos of Genotype p/p

Injection conditions	Embryos injected	Embryos transplanted	Pregnant recipient	Pups born		Embryo survival[a] (%)	Chimeras
				Alive	Died		
With pronase	72	60	7	17	3	33 (28)	0
Without pronase	64	42	6	8[b]	2	24 (19)	0

[a]Survived to term in pregnant recipients.
[b]Of these, three died 2 days after birth.

clearcut differences between the lines. The ES cells of the majority of the studied lines differentiated into mesoderm derivatives (fibroblast-like cells) and, to a less extent, into epithelial-like cells, putative endoderm derivatives. Differentiation into neural cells occurred only in the ES cells of lines MES8 and partly MES9. However, under these in vitro conditions, epithelium of secretory or keratin type did not appear. This may be a sign of restricted pluripotency of the MES lines, although the restriction was line dependent. Nevertheless, the inappropriateness of the in vitro culture conditions used for differentiation is a possibility not to be dismissed.

Third, of the four lines of ES cells studied, injected subcutaneously into athymic mice, only MES1 gave rise to tumors. Histological evaluation revealed a paucity of cell types in the tumours. They contained mainly fibroblast-like cells and rarely smooth muscular cells and stray islets of epithelial tissue. No neural elements or epithelium of keratin type was observed. These results are in agreement with those obtained in tests of this line under conditions of in vitro differentiation.

As was noted above, when cultured in vitro without feeder, MES1 cells differentiated into fibroblast-like vimentin-positive cells. The paucity of cell type composition of the tumors may be a sign of the restricted pluripotency of MES1 cells. The fact that no chimaeric offspring developed from those blastocysts into whose cavity we introduced MES1 cells supports our conclusion concerning the restricted pluripotency of MES1 cells.

Thus it is feasible to isolate ES cells from mink blastocysts and to culture them for a long time (more than 30 passages). The cause of the reduced pluripotency of mink ES cells is unclear. Inappropriate culture conditions may affect adversely pluripotential capacity, or perhaps restricted pluripotency is a feature of the cells from which we derived series MES. It may be advantageous to use embryos at the stage of morula or early blastocyst to obtain in a more consistent way highly pluripotential lines of ES cells in mink.

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